Evaluation of the efficacy of DNA macro chips in early detection of septicemia in children receiving cancer chemotherapy

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ABSTRACT

Title of the abstract

Evaluation of the efficacy of DNA Macrochips in early detection of septicaemia in children receiving cancer chemotherapy

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OBJECTIVES

To assess the sensitivity, specificity and predictive value of DNA Macrochips in the diagnosis of septicaemia and compare the results of the DNA Macrochip with blood cultures and severity of illness in children with various malignancies during febrile episodes.

METHODS

This prospective study was conducted in the Pediatric Hematology – Oncology unit of Christian Medical College, Vellore. Children aged less than 15 years, who were on cancer chemotherapy and with fever were included in the study. The sample for the DNA Macrochip analysis was collected from the patients at the time of hospitalization, along with the sample for blood culture. The sample for blood culture was processed in the Microbiology laboratory, Christian Medical College, Vellore, while the sample for DNA macrochip analysis was performed at XCyton Diagnostic Laboratory, Bangalore. In order to standardize the test, selected organisms from pure cultures were added to blood samples from healthy volunteers . These samples were analysed using the DNA Macrochip and the results compared.

Sensitivity, specificity, Positive predictive value and negative predictive value were calculated from the data obtained from the study. The results were also correlated with the clinical profile in terms of the severity of illness in the patients.

RESULTS

We studied 157 febrile episodes in 121 febrile children undergoing chemotherapy for various malignancies and compared the results of blood culture and the DNA Macrochip in this group. Blood culture was positive in 15 cases whereas DNA macrochip identified 120 organisms from 85 blood samples with multiple isolates in 28 cases. Both tests identified same organism only in 4 cases.

Sensitivity and specificity of the DNA Macrochip was calculated using blood culture as gold standard. The sensitivity was 26.7% and specificity was 47.37%, positive predictive value was 5.4% and negative predictive value was 85.13%. These were too low for a diagnostic test, therefore DNA Macrochip concept in it's present stage is not an useful methodology for the detection of septicemia.

In the standardization study where 14 blood samples with known organisms were analysed, the DNA Macrochip identified organisms in all 14 samples including 4 negative controls. In eight samples correct organism was detected, but in four samples additional organisms were isolated. The possibilities of the DNA primer used for PCR amplification cross reacting with the genome of other organisms, thereby giving false positive results as well as the contamination of the blood samples may explain this observation.

In the comparative analysis of the blood culture and DNA Macrochip against foci of infection and severity of illness, it was found that the severity of illness was similar in both groups, suggesting equivalence between the two groups. However, sample sizes were too small to draw any conclusions from this.

INTRODUCTION

Infection is a major cause of morbidity and mortality in pediatric oncology patients [1]. Febrile episodes occur in approximately one-third of neutropenic episodes in children with chemotherapy-induced neutropenia [2]. The possible infectious origin of fever is a central point in the management of neutropenic patients. A rapid microbiological diagnosis could therefore confirm an infectious cause of fever (thereby excluding non-infectious causes) and help in the choice of a specific therapy.

The current gold standard for the detection of bacterial pathogens in blood is blood culture. However, all blood culture systems suffer from several limitations, such as lack of rapidity and low sensitivity, especially when the patient has already received antibiotics and when fastidious micro-organisms are involved [3]. From this perspective, the diagnosis of bloodstream infections could prove really challenging in hemato- oncological patients, who routinely receive prophylactic antibiotics and whose blood cultures therefore often remain negative [4,5,6,7].

Even after the detection of growth in cultured blood (usually not before 6–12 h of incubation), conventional blood cultures require at least a further 24–48 h for the definitive identification of the pathogen and the evaluation of its sensitivity to antibiotics [3,8]. Other parallel approaches are therefore needed, and among them well-designed molecular assays could prove really useful.

Several molecular techniques have already been successfully used in routine microbiology laboratories for direct detection of viral, bacterial, mycotic and protozoan pathogens [9,10]. However, their use on whole blood samples for detection of sepsis has been hampered by

several factors, including insufficient sensitivity, presence of PCR inhibitors in blood, and the difficulty of setting up an assay capable of detecting a wide range of potential pathogens [3].

The DNA Macro Chip is a new concept, that allows for the simultaneous identification of multiple organisms like bacteria, viruses, fungi and parasites in a single test from a single sample. It involves the concept of syndrome based diagnosis, which allows for simultaneous detection of all probable causative agents which can cause sepsis, obviating multiple sequential tests and loss of time.

The Pediatric Hemato-oncology unit in this Hospital sees over 350 new cases every year and has over 1200 inpatients admissions. Approximately 15%-20% of these admissions are for febrile infections. Blood culture positivity in paediatric oncology patients in our unit from two previous studies has been found to be about 25%.

We proposed to evaluate the role of the DNA Macrochip in pediatric oncology patients who are admitted with febrile episodes and compare it to the gold standard *i.e.* blood culture. We undertook this prospective study to evaluate the sensitivity and specificity of the DNA Macrochip in the detection of septicaemia.

AIMS AND OBJECTIVES

Aim of the study

To evaluate the efficacy of DNA Macrochips in early detection of septicaemia in children receiving cancer chemotherapy.

Objective of the study

1. To assess the sensitivity, specificity and predictive value of DNA Macrochips in the diagnosis of septicaemia

2. To compare the results of the DNA Macrochip with blood cultures in children with various malignancies during febrile episodes

3. To assess whether the DNA Macrochip can be used as an added / alternate modality in detecting bacteremia during febrile episodes in children undergoing cancer chemotherapy.

METHODOLOGY

Study Design

This prospective study was conducted in the Department of Pediatric Hematology – Oncology unit of Christian Medical College, Vellore from December 2009 to November 2010.
One hundred and fifty seven (157) febrile episodes in 120 children undergoing chemotherapy for various malignancies were included in this study.

Children were recruited to this study when they presented with fever to emergency department or in-patient or out-patient ward and after obtaining parental consent.

Sample Size

The sample size for the study was calculated using the formula $4\ pq/d^2$

Since there is no previous data or literature available on DNA Macrochip, considering the fact that this is a diagnostic test in which a high sensitivity and specificity is expected, we aimed for a sensitivity of 90%. The precision was taken as 10. Based on this calculation, we arrived at a sample size of 36 positive blood cultures.

Blood culture postivity in our centre, from previous two studies is about 25%.

Based on these, a minimum of144 children with febrile illnesses need to be recruited for this study.

Inclusion criteria

Children aged less than 15 years, who were on cancer chemotherapy and with fever were eligible for inclusion in the study if they met all the inclusion criteria .

1. Children undergoing chemotherapy for malignancy
2. Fever defined as temperature more than or equal to 101^0F on one occasion or 100.4^0F on two occasions within 4 hours.
3. Informed consent obtained from the parents.

Exclusion criteria

1. Children who do not fulfill the above criteria

2. Children whose parents do not give consent to participate in the study

Patient selection

Participant selection was done from a consecutive series of children who met the inclusion criteria and were admitted with febrile illness.

Patient evaluation

Children who fulfilled the inclusion criteria were evaluated as follows. Initial evaluation included detailed medical history and physical examination, a complete blood count and blood culture. Other investigations like Chest XRay, throat swab, urine culture, pus swab wee done depending upon the indication. Blood culture samples taken from either peripheral vein or percutaneous central lines or port-a-cath.

A proforma (Annexure no:2) was filled with details of patient regarding the demographic data, date of admission, date of discharge, diagnosis, duration of fever, maximum rise of temperature, focal symptoms and signs, antibiotic regimen used, any change made to these antibiotics, investigation reports including blood counts and microbiological

details if positive, Chest X ray report, results of the other investigations evaluating cause of fever and outcome of each episode.

Patient management and monitoring

All the children were admitted and they were started on broad spectrum intravenous antibiotics. In children without an obvious focus of infection and did not respond to the fist line antibiotics (Cefotaxime and Gentamicin) within 72 hours or worsening of symptoms, second line antibiotics (Cefepime and Augmentin) were started. Children who did not respond to 2nd line antibiotics within 72 hours or clinical deterioration, third line antibiotics (Vancomycin and Meropenam) along with systemic antifungal therapy (Amphotericin) were started. In those with culture proven infections, antibiotics were changed according to culture sensitivity results.

Children with ear infection were started with Ceftazidime and Amikacin initially and were changed according to the culture and sensitivity pattern. Children with localized abscess were started on Cloxacillin as initial treatment.

Fluconozole, amphoteicin and or acyclovir were added based on the suspicion of fungal and or Herpes infections respectively.

Children were monitored in the Hospital daily until fever subsided.

Sample collection and processing

The sample for the DNA Macrochip analysis was collected from the patients at the time of hospitalization, along with the sample for blood culture and other routine blood tests.

The sample for blood culture was processed in the department of Microbiology, Christian Medical College, Vellore. The appropriate expertise and quality control measures already exist in the department of microbiology since this is a routine diagnostic test performed in the hospital.

The sample for DNA macrochip analysis was collected and transported to the XCyton Diagnostic Laboratory, Bangalore, where DNA extraction and the multiplex PCR was performed. After collection of the sample and during transport, the sample was stored in room temperature.

The index test was interpreted independently of the reference standard and without knowledge of the results, to minimize bias and in the light of clinical data of the participants.

The results of the index test were not considered for the management of the study subjects.

Standardization of the test

In order to standardize the test, we also collected blood samples from healthy volunteers with no clinical evidence of any infection. To these blood samples, selected organisms from pure cultures available in the Microbiology laboratory in our centre were added in various dilutions. These samples were coded and sent to the XCyton laboratory, Bangalore for analysis using the DNA Macrochip.

Statistical methods

Sensitivity, specificity, Positive predictive value and negative predictive value were calculated from the data obtained from the study.

The data was entered into a Microsoft Windows Excel work sheet.

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 16.0 for Windows (SPSS Inc, Chicago, Ill)

DNA Macro Chip

DNA Macro Chip is a technology developed by XCyton Diagnostic Laboratory, Bangalore. The product is still undergoing trials at various centres in India and is not marketed commercially. Since the product is still in the trial phase, we donot have any published literature on the efficacy of this test.

XCyto Screen DNA Macro Chip is a new technology that allows for the simultaneous identification of multiple organisms like bacteria, viruses, fungi and parasites in a single test from a single sample.

It involves the concept of syndrome based diagnosis, which allows for simultaneous detection of all probable causative agents which cause the syndrome, obviating multiple sequential tests and loss of time. Till date, there are several similar kits available for specific infections like eye and CNS infections. These have been standardized and are currently available commercially.

The DNA Macrochip used in this study is the first such template which aims to detect the presence of sepsis causing organisms from blood samples. Since there are dozens of organisms which include Gram positive and Gram negative bacteria as well as fungi which are known to cause sepsis, the ideal test should be able to identify multiple organisms.

The technology involves extraction of the genetic material of the causative agent from the given specimen and simultaneously amplifies the “Signature genes” of all the causative agents. To identify the causative agent’s signature gene which got amplified, the signature gene (amplified product) is denatured and allowed to re-nature to its complimentary “gene sequence” that is embedded on the DNA Macro Chip. Further a colorimetric reaction is performed to get a visual signal.

The test basically involves 4 steps

1. Extraction of the DNA of the organism from the blood sample
2. Amplification of the DNA using PCR and virulence gene specific primers
3. Hybridisation of the amplified DNA with the complementary strands embedded in the DNA Macrochip platform
4. Reporting of the tests based on the visual signal from the colorimetric reaction, which will be present in case of a positive reaction and identification of the organism by matching it with the preset grid.

Organisms which can be detected by the DNA Macrochip technology at present

Serial No	Organism screened
Gram Positive Organisms	
1	*Staphylococcus aureus*
2	*Streptococcus species (other than S.pyogenes and S.pneumoniae)*
3	*Streptococcus pyogenes*
4	*Streptococcus pneumoniae*
5	*Enterococcus species*
Gram Negative organisms	
6	*Klebsiella pneumoniae*
7	*Salmonella typhi*
8	*Proteus mirabilis*
9	*Neisseria meningitides*
10	*Haemophilus influenzae*
11	*Pseudomonas aeruginosa*
12	*Acinetobacter baumanii*
13	*Bacteroides fragilis*
Fungus	
14	*Candida species*
15	*Aspergillus species*

Detailed test procedure of the DNA Macrochip and the quality control measures adopted in the laboratory is given in annexure 1.

REVIEW OF LITERATURE

Febrile neutropenia

Fever is the principal and sometimes the only manifestation of serious infection in the immunocompromised patient [11,12]. Fever may be the first manifestation of a life-threatening infection, particularly during periods of neutropenia. Febrile episodes occur in approximately one-third of neutropenic episodes in children with chemotherapy-induced neutropenia [4].

Fever is a manifestation of the release of proinflammatory cytokines (interleukin-1α, interleukin-1ß, interleukin-4, interleukin-6, and tumor necrosis factor α) from macrophages, lymphocytes, fibroblasts, epithelial cells, and endothelial cells as a consequence of infection or inflammation[13]. Although endogenous pyrogens are classically thought to originate from polymorphonuclear leukocytes, patients with profound neutropenia have high fevers when they have infections, so reservoirs of pyrogens other than neutrophils are also important.

Although a number of fever patterns have been associated with various infectious or noninfectious illnesses, no pathognomonic pattern or degree of fever has been clearly associated with a specific infection in immunocompromised patients. There is also no pattern of fever that can be used to rule out a noninfectious cause[12]. Furthermore, patients who are profoundly immunocompromised can have serious local or systemic infections in the absence of fever. Fever can also be suppressed or muted by immunosuppressive agents that may be part of the therapeutic regimen, especially steroids and nonsteroidal antiinflammatory agents.

At the time of diagnosis, fever is present in almost 80% of children with acute Leukemia [14]. Often, no obvious focus is found and it may be due to disease fever. Classically, this fever is cytokine mediated and it defervesces within a day or two after starting chemotherapy.

Fever and infection in the setting of neutropenia can be life threatening, if left untreated [15]. Because of the blunted inflammatory response in patients with neutropenia, the signs and symptoms of infection can be minimal, so a heightened index of suspicion for infection is essential. Infection advances rapidly in the neutropenic host and bacteremia can progress to septic shock and death within a few hours.

Cofactors for severe infection include the degree of neutropenia, its duration, and whether there are other perturbations in the host defenses. Patients who have neutropenia after cytotoxic chemotherapy nearly always have breaches of physical defense barriers, typically with oral and gastrointestinal mucositis, which permit changes in colonization as well as serving as nidi for local infection and entry points for systemic invasion[16]. Such patients are also likely to have alterations in cellular immunity as well as hypogammaglobulinemia, which make these patients among the most vulnerable to acute infections[17].

Febrile neutropenia is defined as a single oral temperature of 38.3 degree Celsius / 101 degree Fahrenheit or 38 degree Celcius / 100.4 degree Fahrenheit for atleast one hour with an absolute neutrophil count of 500/mm3 or 1000/mm3 with a predicted decline to 500/mm3 [14].

Patients with neutropenia can be divided into low- and high-risk groups on the basis of the projected duration of neutropenia. Patients at low risk (generally those with solid tumors and those who have received less intensive chemotherapy regimens) have had neutropenia for no more than 10 days and usually have excellent outcomes, rarely complicated by secondary

infectious complications[11]. In contrast, patients at high risk (those who have had neutropenia for more than 10 days) are vulnerable not only to acute bacterial infections but also to second or even multiple infectious complications from bacteria, fungi, viruses, or parasites.

Organisms Associated with Infection

The spectrum of organisms responsible for infectious complications in immunocompromised hosts is daunting, since virtually any organism can become invasive if host defenses are severely impaired[11,12]. The predominant organisms are influenced by the patient's treatment regimen as well as by where the patient resides and receives care.

Bacteria represent the immediate threat to most immunocompromised hosts. During the past two decades, there have been changes in the dominant organisms responsible for infection in immunocompromised hosts with neutropenia [11,18,19,20]. Gram-positive organisms, especially the coagulase-negative staphylococci, have emerged as the leading cause of acute bacterial infections associated with fever and neutropenia in patients in the United States and western Europe, which may be partly due to the increased use of indwelling intravenous-access devices, although this trend began before the routine use of these devices. In developing countries gram-negative organisms, including *Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella* species, still predominate.

In a study conducted by Hakim et al[21] in 337 febrile neutropenic episodes, infection was proven in 86 (25%) and probable in 75 (22%). Bacteremia accounted for most (41) of

the proven bacterial episodes, with *Streptococcus viridans* (13), *Pseudomonas spp* (6) and *E. coli* (6) the most frequently isolated organisms.

Methods of detection of Bacteremia

Blood culture

Blood cultures remain the standard method for detecting bacteremia in the evaluation of sick infants and children[22,23]. The isolation of an organism confers many advantages, including the optimal choice and duration of antibiotic treatment[24]. The exclusion of bacteremia is also important, because it may enable the cessation of antibiotic treatment and consequently reduce the length and cost of hospital stay, as well as decreasing the development of antimicrobial resistance[25,26] . The ability to exclude bacteremia on the basis of a negative blood culture result depends on the sensitivity and negative predictive value of this test.

However, this technique has some limitations, including

1) it takes a long time for growth of an organism (24-48 hours)

2) it can have unacceptably low sensitivities (40%) in many clinical situations and its sensitivity might be less if the patients have had prior antibiotic treatment[27]

Hakim et al[21] reported that the median time to positivity of blood cultures was 12 hours (range, 5.4-143.7) with 93% positive within 24 hours of incubation.

Cockerill et al[28], in a study evaluating 37,568 blood cultures found that two blood cultures detected only 80% of bloodstream infections and that three blood cultures detected 96% of episodes.

Lee et al[29], studied 629 unimicrobial episodes with ≥3 blood cultures obtained during the first 24-h period. They found that 460 (73.1%) were detected with the first blood culture, 564 (89.7%) were detected with the first two blood cultures, 618 (98.2%) were detected with the first three blood cultures, and 628 (99.8%) were detected with the first four blood cultures.

Blood Culture Systems

***Manual detection systems*:** The simplest blood culture system consists of bottles filled with broth medium and with a partial vacuum in the headspace. After inoculation, the bottles are incubated with or without agitation and are periodically examined for macroscopic evidence of growth. Such evidence consists of hemolysis, turbidity, gas production, "chocolatization" of the blood, and the presence of visible colonies or a layer of growth on the fluid meniscus.

Manual systems are too labor-intensive to be practical for most laboratories that process large numbers of blood cultures, since it is needed to manually inspect each bottle daily for macroscopic evidence of growth and to perform blind and/or terminal subcultures. Performing blind and/or terminal subcultures also increases the likelihood that technologists will be exposed to patient blood and adds to laboratory cost.

***Automated detection systems*:** To make processing blood cultures more efficient, several manufacturers have developed and marketed a number of automated blood culture systems over the past 20 years.

***BACTEC radiometric blood culture systems*:** The first commercial automated blood culture system was the radiometric BACTEC system (Becton Dickinson Microbiology Systems).

This system detected microbial growth by monitoring the concentration of CO2 present in the bottle. In this system, metabolic precursors labelled

with 14C are included in the broth medium; as microorganisms grow, they liberate 14CO2 in the broth, which diffuses into the bottle headspace atmosphere. The headspace atmosphere is periodically sampled, and the amount of 14CO2 is measured. This quantity is related to a "growth index"; a growth index exceeding a predefined threshold is considered evidence of microbial growth and prompts a Gram stain and subculture of the blood-broth mixture

***BACTEC nonradiometric blood culture systems* :** Conceptually and operationally similar to the radiometric systems, these instruments use infrared spectrophotometry to detect

CO2 in samples of the bottle headspace atmosphere. The newer systems hold more bottles,require shorter monitoring times

Continuous-monitoring blood culture systems (CMBCS **:** The most important technological advance in blood cultures during the past 20 years has been the development of continuous-monitoring blood culture systems (CMBCS). CMBCS differ from other automated blood culture systems in several ways. These instruments electronically monitor bottles for evidence of microbial growth on a nearly continuous basis, typically once every 10 min. Data collected from this monitoring are transmitted to a microcomputer, where they are stored and analyzed. CMBCs detect positive results 1 to 1.5 days earlier than previously used conventional blood culture systems.

BacT/Alert blood culture system **:** BacT/Alert (Organon Teknika Corp.) was the first commercial CMBCS [30]

Within the base of each bottle is a solid sensor that undergoes a color change as the CO2 concentration in the broth medium increases. Light-emitting and -sensing diodes are incorporated in the base of each cell in which the bottles reside. As the sensor changes color, the amount of light reflected off the sensor increases and is quantified as an increasing voltage signal. These signals are recorded by a microcomputer and are analyzed by an algorithm. The algorithm recognizes three criteria as evidence of microbial growth: an initial reading that exceeds a predefined threshold (analogous to the BACTEC growth indices), a sustained linear increase in CO2 concentration (production), or an increase in the rate of CO2 production. Currently, standard aerobic

and anaerobic (accepting blood inocula of up to 10 ml), pediatric, and FAN aerobic bottles are available [30].

Limitations of Blood Culture

While a number of clinical and technical factors may affect the isolation of the infecting organism, the volume of blood sampled is the most critical factor in the detection of bloodstream infection[3]. The number of organisms present in bacteremia is frequently low, often less than 10 colony-forming units per millilitre (CFU/mL) [31,32]. There is a direct relationship between blood volume and yield, with an approximately 3% increase in yield per millilitre of blood cultured [32]. National standard laboratories in Europe recommended that at least 20 to 30 mL of systemic blood be cultured from adults [33].

Contamination of blood cultures giving a 'false-positive' result remains a significant problem that can limit diagnostic utility in the critically ill and is closely associated with poor patient-sampling techniques. Documented rates of contamination vary considerably between institutions, from 0.6% to over 6% [34]. Repeating blood culture sets increases sample volume and pathogen yield in the setting of bloodstream infection but is primarily recommended to assist in the recognition of contamination.

The sensitivity of blood cultures decreases greatly when taken after the initiation of antimicrobial therapy [35,36]. The use of prophylactic antibiotics and antifungal agents in immuno-compromised neutropenic patients makes diagnosis challenging as blood cultures remain negative in many cases [37]. Such patients are also at considerable risk of

acquiring infection caused by slow-growing and fastidious organisms, including fungi, for which blood cultures are poorly sensitive.

Studies reported in the 1970s, 1980s, and early 1990s suggested that two to three blood cultures from adults obtained during a 24-h period could detect >99% of all bloodstream infections [38,39,40,41].

However, a 2004 study from the Mayo Clinic using the BACTEC 9240 CMBCS found that two blood cultures detected only 80% of blood stream infections, that three detected 96% of blood stream infections, and that four were required to detect 100% of blood stream infections [28].

The detection and identification of microorganisms based on traditional culture-based methods take time, because of the significant time lags between patient sampling and results. This can take 2 to 3 days for bacteria and much longer for other fastidious organisms. Hence the time required to prove absence of infection by culture methods can exceed that of a treatment course of antibiotics [42].

Therefore, although blood cultures remain at the heart of the sepsis care guidelines, emerging alternative technologies aimed at complementing the deficiencies of culture, particularly related to improving time-critical diagnostics, are being investigated.

Newer tests for detection of Bacteremia

In the last few years many new techniques have been introduced for the early diagnosis of bacterial infections.

These new tests can be subdivided into three broad headings

I. Demonstration of indirect evidences of bacterial infection by identifying serological markers of systemic inflammatory response syndrome
II. Direct demonstration of bacteria by identifying bacterial genomes
II. Rapid bacterial culture methods

Indirect evidence of Bacterial infection by identifying serological markers

Sepsis is considered when there is a systemic response to a possible infection, termed as systemic inflammatory response syndrome (SIRS).

The quest for surrogate biomarkers to define SIRS has identified several potential candidates. Markers such as procalcitonin, C-reactive protein (CRP), tumor necrosis factor alpha (TNF-a), and several interleukins have appeared promising in initial studies.

1.Procalcitonin (PCT)

PCT is a new marker that has been associated with inflammation and sepsis. It is a 116-amino-acid protein that is the precursor to calcitonin. The PCT plasma level in healthy

individuals is low; usually below 0.1 ng/ml. The levels have been shown to rise with severity of sepsis.

The sensitivity of PCT in initial determinations for the diagnosis of sepsis has been reported to vary from 61% to 85%, increasing to 72–100% within the subsequent 24 h. PCT specificity was found to vary in initial determinations from 50% to 97% and ranged between 63% and 97% within the next 24 hr. [43]

PCT had the highest sensitivity and specificity for differentiating SIRS from sepsis. It has been found to be a more reliable marker in the diagnosis of sepsis than other measures. However, in a study conducted in our unit in 2008[44], it was seen that the mean PCT values are not significantly different among those with bacteremia and no bacteremia and the sensitivity and specificity were not high to recommend it as a stand alone diagnostic marker.

2. C-reactive protein (CRP)

The acute phase protein CRP is released from the liver in response to proinflammatory cytokines and thought to recruit monocytes in early infection.

Sensitivity and specificity of CRP levels in older children has been poor. However, in newborns CRP specificity averages 90%. Serial CRP levels are useful in the diagnostic evaluation of neonates with suspected infection. Two CRP levels <1 mg/dL obtained 24 hr apart, 8 to 48 hours after presentation indicate that bacterial infection is unlikely.

The sensitivity of a normal CRP at the initial evaluation is not sufficient to justify withholding antibiotic therapy. The positive predictive value of elevated CRP levels is low, especially for culture proven early-onset infections.[45]

Demonstration of bacteria by identifying bacterial genomes

During the past decade, there has been unprecedented progress in molecular biology as well as in the application of nucleic acid technology to the study of the epidemiology of human infection. These include

1. Polymerase chain reaction
2. Nucleic Acid Hybridization
3. RNA Typing or Ribotyping
4. PCR Ribotyping
5. Pulsed-Field Gel Electrophoresis
6. Clamped Homogeneous Field Electrophoresis
7. Multilocus Sequence Typing
8. Fluorescence-Based Amplified Fragment Length Polymorphism
9. DNA Sequencing and Molecular Evolutionary Analysis

1. **Polymerase Chain Reaction assay (PCR)**

PCR is currently the most widely used nucleic acid amplification and detection method. It has been found to have a substantial impact on the diagnosis of infectious diseases[46,47.] This method has the ability to amplify minute amounts (less than 3 copies) of specific microbial DNA sequences in a background mixture of host DNA making it a powerful diagnostic tool[48] . Nucleic acid amplification is performed in a thermocycler, which is an instrument that can hold the assay's reagents and allows the reactions to occur at the various temperatures required.

In the initial step of the procedure, nucleic acid (*e.g.,* DNA) is extracted from the microorganism or clinical specimen of interest. Heat (90ºC-95ºC) is used to separate the extracted doublestranded DNA into single strands (denaturation). Cooling to 55ºC then allows primers specifically designed to flank the target nucleic acid sequence to adhere to the target DNA (annealing).

Following this, the enzyme Taq polymerase and nucleotides are added to create new DNA fragments complementary to the target DNA (extension). This completes one cycle of PCR. This process of denaturation, annealing and extension is repeated numerous times in the thermocycler. At the end of each cycle each newly synthesized DNA sequence acts as a new target for the next cycle, so that after 30 cycles millions of copies of the original target DNA are created. The result is the accumulation of a specific PCR product with sequences located between the 2 flanking primers[49]

Advantages of PCR

1. PCR-based methods can detect as few as 10 to 100 copies of bacterial genome in clinical samples.
2. The sample does not have to be highly purified, and even the starting DNA may be partially degraded as long as the target sequence (~100– 1000 bp) is intact.
3. The rapidity of PCR makes it possible to generate detectable amounts of amplified target nucleic acid within hours, compared with several days by probe hybridization procedures and often weeks for the identification of many fastidious organisms by culture.

PCR assays that are currently available commercially for use in diagnostic laboratories include tests for the detection of *Chlamydia trachomatis*, *C. pneumoniae*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae* and *Neisseria gonorrhoeae*[50] Multiplex PCR-based assays have been developed and have the advantage of detecting multiple pathogens in a single PCR reaction. These have been used to detect common bacterial causes of respiratory tract infections, bacteremia and meningitis.

Limitations of PCR

Despite the obvious advantages to these newer procedures, there may be potential limitations to DNA amplification technology in the diagnostic laboratory.

1. The accuracy and reproducibility of PCR assays depend on the technical expertise and experience of the operator.
2. The extreme sensitivity of PCR paradoxically leads to one of its major drawbacks—the occurrence of false-positive results. Very small amounts of the amplified target sequence, of which up to 10 copies can be present in a single PCR solution, can contaminate laboratory equipment or reagents. The PCR product can even spread as airborne droplets in areas of sample or reagent preparation. This contaminating DNA can then serve as a template for further amplification, resulting in false-positive results in subsequent samples. For this reason laboratories should have separate rooms for different steps of the PCR procedure and must follow stringent quality control measures to prevent contamination or carry-over.
3. False-negative test results may occur because of the presence of substances in the specimen that inhibit nucleic acid extraction or amplification. Certain specimen types like blood are more likely to contain such inhibitors.
4. The assays may also lack sensitivity if there is a low inoculum of the microorganism present in the clinical specimen. This may be exacerbated if an inadequate sample or very small specimen volume (*i.e.*, < 20 µL) is available for testing.

5. Another source of error is the detection of nonviable organisms by PCR, since it amplifies only a portion of the microbe's genome. In such instances, the detection of complementary DNA by reverse-transcription PCR of messenger RNA encoded by the pathogenic organism can serve as evidence of active infection.
6. Interpretation of nucleic acid amplification test results is not always clearcut. For example, assays may detect the residual DNA of a pathogenic microorganism even after successful treatment, and it is not clear whether this represents the presence of a small number of viable organisms or amplified DNA from nonviable organisms. Therefore, PCR tests should not be used to monitor the effectiveness of a course of therapy.
7. Finally, the performance of a PCR assay is generally more expensive than conventional diagnostic laboratory methods.

2. Nucleic Acid Hybridization

Nucleic acid hybridization in its simplest form can be used in the detection of microorganisms or specific resistance genes. It confirms the results of microbial cultures or even detects organisms in clinical samples. This may require the extraction of DNA or, in some cases, RNA from a clinical sample (body fluid, peripheral white cells, aspirate or

scraping, or fresh tissue). A labeled synthetic nucleic acid probe often less than 30 bases long can detect the presence of target nucleic acids (oligonucleotide probe). The oligonucleotide probes directly identify microbial genetic sequences in contrast to conventional immunologic tests and Western blot techniques, which pick up microbial gene products or proteins. Molecular hybridization has greater sensitivity and specificity than these conventional techniques.

3. RNA Typing or Ribotyping RNA typing or ribotyping is a chromosomal detection technique. Ribosomal genes are present throughout the chromosomes of bacteria: the sequences of DNA between the ribosomal genes vary in length.

The digestion of chromosomal DNA by endonuclease produces random fragment polymorphic patterns when probed with ribosomal RNA.

For this method, *E coli* 16S ribosomal RNA serves as a probe for the endonuclease-digested chromosomal DNA. Because chromosomal nucleotide patterns usually vary from strain to strain but not within a strain, this technique can identify organisms and differentiate strains.

The technique has application in the typing of *HaemophilusInfluenza*, *Pseudomonas capacia*, *E coli, Salmonella typhi,* and *Providencia stuartii*.[51]However, ribotyping is not as useful for gram-positive organisms.

4. PCR Ribotyping

PCR ribotyping is the analysis of banding patterns obtained by gel electrophoresis of PCR amplified fragments of the 16S to 23S ribosomal RNA intergenic spacer regions.
It has considerable advantages in terms of speed and technical ease, and is useful in detecting and typing *C difficile* strains.[52]

5. Pulsed-Field Gel Electrophoresis This is a widely used technique for analyzing a large amount of chromosomal DNA found in large bacterial chromosomal fragments generated by endonuclease digestion.[53]

6. Clamped Homogeneous Field Electrophoresis

This method was developed to compare large chromosomal fragments generated by restriction endonuclease digestion. It is a form of pulsedfield gel electrophoresis, which is an easy way to compare isolates of a species.
Clamped homogeneous field electrophoresis helps in the analysis of vancomycin resistant enterococci. This technique can type other bacteria, mycobacteria, and yeast as well.

7. Multilocus Sequence Typing

In principle, this is the genome-based version of the conventional method of multilocus enzyme electrophoresis. It helps in the typing of various bacterial species by identifying DNA alleles from various organisms, including *Campylobacter jejuni, and Enterococcus faecium*.

The method involves PCR amplification and the nucleic acid sequencing of multiple internal fragments of genes.

The advantages of this approach are that the culturing of pathogenic microorganisms is avoided, as their gene fragments are amplified directly from biologic samples, and that the sequencing data are unambiguous and easy to standardize.

8. Fluorescence-Based Amplified Fragment Length Polymorphism

This is a novel assay based on the fluorescent analysis of an amplified subset of restriction fragments. The fluorescence-based amplified fragment length polymorphism assay involves the selective PCR amplification of restriction fragments from a total digest of genomic DNA. It has been useful in the study of vancomycin - resistant *enterococci*.[54]

9. DNA Sequencing and Molecular Evolutionary Analysis

The sequencing of DNA refers to the enumeration of individual nucleotide base pairs along a linear segment of DNA. Single-stranded or double stranded DNA generated by PCR can be used directly for DNA sequencing. Automation has made it possible to double the lengths of readable DNA sequences obtained after a single sequencing run to more than 400 to 800 bp.

In summary, we have several techniques available for the detection of pathogens from blood as well as other specimens. Blood cultures remain the standard method for detecting bacteremia, with the advantages that it is a time proven technique which is

easily available. However, it also has its disadvantages in terms of low sensitivity, especially when single cultures are done and the time taken for results, which is especially important in time critical settings such as infection in immunocompomised patients.

Although there are a number of molecular techniques available for the detection of pathogens from blood samples, most lack sensitivity and are expensive and cumbersome in the clinical setting.

Hence, we need a technique which is at least as sensitive as culture, allows a faster identification of pathogens, not influenced by ongoing therapy and ability to identify multiple organisms from the same sample in single test.

The DNA Macrochip is one such technique which involves the concept of syndrome based diagnosis, which allows for simultaneous detection of all probable causative agents which can cause sepsis, obviating multiple sequential tests and loss of time.

There are other techniques such as the DNA Microarray technique, which in principle is similar to the DNA Macrochip, i.e PCR amplification followed by hybridization. However the two techniques differ in the type of gene probe used for the PCR amplification. The gene probe used in the DNA Macrochip is an unique one which is specific to the virulence coding genes of the organism being tested, where as the DNA Microarray probes are based on the detection of the 16S and 23S ribosomal genes, which is theoretically less specific to particular organisms as compared to virulence specific genes.

RESULTS

One hundred and fifty seven (157) febrile episodes from 121 children were included in this study

Age and Sex distribution

The study was conducted on children aged upto 15 years. The age and sex distribution of the children included in the study is shown table 1.

Table 1. **Population distribution**

Age (years)	Boys	Girls
0 to 5	36 (41.4%)	15 (44.1%)
6 to 10	32 (36.8%)	8 (23.5%)
11 to 15	19 (21.8%)	11 (32.3%)
Total	**87**	**34**

There were 87 boys and 34 girls in the study group. The mean age of the study population was 7.4 years, median age was 7 years and the range was 1 to 15 years.

Types of malignancies in the study population

The study population comprised of children with various malignancies, admitted with febrile illness. The types of malignancies and the frequency of their occurrence in the study population is listed in table 2.

Table 2. **Distribution of malignancies among the study population**

Serial No	Type of Malignancy	Number	Percentage
1	Acute Leukemia	81	66.9
2	Hodgkin's lymphoma	8	6.6
3	PNET / Ewing sarcoma	7	5.8
4	Medulloblastoma	6	4.9
5	Osteosarcoma	4	3.3
6	Neuroblastoma	4	3.3
7	Wilm's tumour	4	3.3
8	Rhabdomyosarcoma	2	1.6
9	Burkitt's lymphoma	2	1.6
10	Peripheral nerve sheath tumour	1	1.6

11	Anaplastic ependymoma	1	1.6
12	Clear cell sarcoma	1	1.6
	Total	**121**	**100**

Table 3. **Absolute neutrophil counts and focus of infections**

ANC (/ mm^3)	**LRI**	**Mucositis**	**GIT infections**	**Local infection**	**No focus**
0 to 500 (n = 108, 68.8%)	21 (19.4%)	19 (17.6%)	15 (13.9 %)	5 (4.6%)	54 (50%)
500 to 1000 (n = 10, 6.4%)	1 (10%)	0	1 (10%)	1 (10%)	8 (80%)
> 1000 (n = 39, 24.8%)	9 (23.1%)	2 (5.1%)	1 (2.6%)	5 (12.8%)	27 (69.2%)
Total*	**31**	**21**	**17**	**11**	**89**

* Some children had more than one focus of infection. Hence the disparity in the total number of patients in this table

Of the 157 febrile episodes studied, 108 had an absolute neutrophil count of less than 500. Ten patients had ANC ranging from 500 to 1000 and 39 patients had ANC more than 1000.

Eighty nine (89) children had no obvious focus of infection. Remaining 68 children had some foci of infection ; these were LRI (31), mucositis(21), GIT infection(17) and local infection (11). Eleven children had more than one focus of infection. The category local

infections included cellulitis in three patients, ear infections in four patients and throat infection in four patients.

There was no statistically significant correlation between ANC and focus of infection in this group.

Blood Culture results in the study population

Blood cultures were taken from all children included in the study at presentation. Out of the 157 samples sent for blood culture 133 were sterile and 24 cultures grew various organisms. The organisms isolated by the positive blood cultures are given in table 4.

Table 4. **Organisms isolated by blood culture**

Serial No	Organism	Number	Percentage
1	Coagulase Negative Staphylococcus	4	16 %
2	Non Fermenting Gram Negative Bacilli	3	12 %
3	*Pseudomonas aeruginosa*	2	8 %
4	*Escherichia coli*	2	8 %
5	*Alpha hemolytic Streptococcus*	1	4 %
6	*Enterococcus species*	1	4 %
7	*Hemophilus influenzae*	1	4 %
8	Gram negative bacilli	1	4 %
10	*Candida*	1	4 %
11	Anaerobic spore forming organism	1	4 %
12	*Klebsiella pneumonia*	1	4 %
13	Contaminants	7	28 %
	Total	**25**	**100**

Of the blood cultures which were positive, 23 samples showed growth of single organism and one sample grew two organisms – *E Coli and Klebsiella pneumoniae.* Seven cultures were reported as contaminants and these from excluded from the comparative analysis.

Among the organisms grown in blood culture *E coli* and anaerobic spore forming organism cannot be identified by the DNA Macrochip. *E Coli* was grown in 2 cultures out of which one culture had also grown *Klebsiella pneumonia*, which can be detected by the DNA Macrochip. This sample was retained in the study while the other sample which grew *E Coli* alone as well as the one which grew anaerobic spore forming organisms, along with the seven blood cultures which grew contaminants were excluded while doing the statistical analysis.

Thus a total of 15 cultures were included for further analysis

Results of the samples analysed using DNA Macrochip

One hundred and fifty seven (157) blood samples were analysed using the DNA Macrochip, of which 85 (54.1%) samples were reported as positive with isolation of single or multiple organisms and 72 (45.9 %) samples were reported as negative.

Table 5. **Pattern of isolation of organisms by the DNA Macrochip**

Number of positive tests	85
Single organism isolated	57 (67.1%)
Two organisms isolated in same sample	21 (24.7%)
Three organisms isolated in same sample	7 (8.2%)

85 of the 157 samples sent for the DNA macrochip analysis showed positive results. A single organism was identified from 57 samples, two organisms were isolated in 21 of the samples and three organisms were isolated in the same sample on seven occasions. There were a total of 120 isolates, including the multiple isolates in the 157 samples analysed.

Table 6. **Organisms isolated by the DNA Macrochip**

Serial			

number	Organism	Number	Percentage
1	*Acinetobacter baumanii*	25	20.8 %
2	*Candida*	25	20.8 %
3	*Pseudomanas aeruginosa*	14	11.6 %
4	*Streptococcus species*	12	10 %
5	*Klebsiella pneumoniae*	11	9.2 %
6	*Staphylococcus aureus*	9	7.5 %
7	*Streptococcus pneumoniae*	8	6.7 %
8	*Aspergillus species*	8	6.7 %
9	*Enterococcus*	5	4.2 %
10	*Bacteroides*	2	1.7 %
11	*Neisseria meningitides*	1	0.8 %
	Total	**120**	**100**

Comparison between blood culture and DNA Macrochip results

We compared the corresponding reports of the blood culture and DNA Macrochip on the same sample. 15 of 24 blood culture isolates were compared with DNA macrochip results. The remaining nine were excluded as there were eight contaminants and one sample which grew *E coli* which cannot be picked up by the DNA macrochip technique.

Table 7. **Comparison between blood culture and DNA Macrochip results**

Result	**Blood Culture**	**DNA Macrochip**
Positive	15	85
Negative	133	72
Comparison of corresponding Positive and Negative samples		
Blood Culture and DNA Macrochip positive with same organism		4
Blood culture sterile and DNA Macrochip negative		63
Blood culture positive and DNA Macrochip negative		4
Blood culture positive and DNA Macrochip positive with different organism		7
Blood culture sterile and DNA Macrochip positive		70

Table 8. **Comparison of organisms isolated by blood culture and DNA Macrochip**

Organism	**Blood culture**	**DNA Macrochip**	**Concordance**

Acinetobacter baumanii	0	25	-
Candida	1	25	1
Pseudomanas aeruginosa	2	14	1
Streptococcus species	0	12	-
Klebsiella pneumoniae	1	11	1
Staphylococcus aureus	0	9	-
Streptococcus pneumoniae	0	8	-
Aspergillus species	0	8	-
Enterococcus	1	5	1
Bacteroides	0	2	-
Neisseria meningitides	0	1	-
Coagulase negative staphylococcus	4	0	-
Non fermenting Gram negative bacilli	3	0	-
Escherichia coli	2	#	-
Gram negative bacilli	1	0	-
Alpha hemolytic streptococcus	1	0	
Hemophilus influenzae	1	0	-
Total	**17***	**120**	**4**

* 16 positive blood cultures out of which one sample had grown two organisms. Of these, the sample which grew *E Coli* as the single organism was not included in the comparative analysis

cannot be detected by the DNA Macrochip

Of the 15 samples which showed growth on the blood culture, only four corresponding samples showed the same organism on the DNA Macrochip. In the samples which correlated, the organisms isolated were; *Pseudomonas aeruginosa, Klebsiella pneumonia, Candida and Enterococcus species.*

Of the remaining samples, there were no isolates in four samples on the DNA Macrochip. Seven samples isolated organisms which were different from those grown

on the corresponding blood culture samples. These were considered as negative in the final analysis.

Table 9. **Sensitivity and Specificity of DNA Macrochip**

	Blood culture Positive	**Blood culture Negative**	**Sensitivity**	**Specificity**
DNA Macrochip Positive	4 (True positive)	70 (False positive)	**26.7 %**	-
DNA Macrochip Negative	11 (False negative)	63 (True negative)	-	**47.37 %**

The sensitivity of the DNA Macrochip was 26.7 % in this study and the specificity was 47.37 %, both of which are low for a diagnostic test. The positive predictive value of the DNA macrochip in detecting bacteremia was very low at 5.4%. The negative predictive value was an acceptable 85.13%

Standardization study comparing the results of the DNA Macrochip with blood samples spiked with known organisms

In order to validate the sensitivity of the DNA Macrochip in a controlled setting, we analysed blood samples taken from healthy volunteers which were spiked with organisms in the Microbiology laboratory in Christian Medical College, Vellore. These samples were sent to the XCyton laboratory, Bangalore for analysis.

Fourteen blood samples from healthy volunteers, collected under controlled situation were inoculated in varying dilutions with pure cultures from Microbiology laboratory.

Of the 14 blood samples, organisms were added to 10 of the samples and 4 samples were used as negative controls, to which no organisms were added.

The organisms added were *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*, Coagulase negative staphylococcus and *Enterococcus*. The samples were coded and sent to the XCyton laboratory in Bangalore for analysis and the results of this analysis was compared.

Table 10. **Comparison of the results of the DNA Macrochip with blood samples spiked with known organisms**

Sample No	Organism added to the blood sample	Organism isolated by DNA Macrochip
1	***Klebsiella pneumonia***	*Staphylococcus aureus ,* ***Klebsiella pneumonia***
2	***Klebsiella pneumonia***	*Staphylococcus aureus ,* ***Klebsiella pneumonia***
3	***Pseudomonas aeruginosa***	***Pseudomonas aeruginosa***
4	***Pseudomonas aeruginosa***	***Pseudomonas aeruginosa***
5	***Staphylococcus aureus***	***Staphylococcus aureus,*** *Acinetobacter baumanni, Pseudomonas aeruginosa*

6	***Staphylococcus aureus***	***Staphylococcus aureus,*** *Pseudomonas aeruginosa*
7	Coagulase negative *Staphylococcus*	*Staphylococcus aureus*
8	Coagulase negative *Staphylococcus*	*Staphylococcus aureus, Enterococcus*
9	***Enterococcus***	***Enterococcus***
10	None	*Aspergillus*
11	***Enterococcus***	***Enterococcus***
12	None	*Aspergillus*
13	None	*Acinetobacter baumannii, Pseudomonas aeruginosa*
14	None	*Candida*

All 14 samples, including four negative controls were positive by DNA macrochip analysis for various organisms. In eight samples the same organism was identified, but in four of these additional organisms were detected by DNA macrochip method.

The DNA Macrochip isolated multiple organisms in six samples – two organism each in five samples and three organisms in one sample.

In the samples in which multiple organisms were isolated by the DNA Macrochip, one of the organisms isolated correlated with the actual organism in the sample. Of the four samples which were used as negative controls, the DNA Macrochip isolated Aspergillus species in two samples and the others showed Acinetobacter baumanii, Pseudomonas aeruginosa and Candida.

Although the sample size of this sub study is too small to comment on the sensitivity of the DNA Macrochip using these results, it is seen that the profile of isolation of organisms is similar to that seen in the actual study in the clinical setting.

Correlation of blood culture and DNA Macrochip results with severity of illness and foci of infection

The gold standard used in this study is the blood culture, which does not have 100% sensitivity and so is an imperfect gold standard. The DNA Macrochip works on the principle of PCR amplification of the bacterial genome, which is theoretically more sensitive in detecting bacterial infections. Hence, it is possible that a number of organisms which are not identified by the blood culture may be detected by the DNA Macrochip, which when compared with the corresponding blood cultures may be labeled as false positives.

We correlated the results of the blood culture and the DNA Macrochip with with focus of infection, antibiotic therapy, duration of hospital stay and outcome as these parameters are indicative of severity of the illness.

Patients with foci of infection were chosen for the analysis as they are more likely to have bacteremia.

This analysis was aimed to compare the clinical profiles of the patients who showed positivity in the blood culture and/or the DNA Macrochip. Such a comparison would help us to know whether the severity of illness in the patients in whom organisms were isolated by the DNA Macrochip is similar to those in whom the gold standard - blood culture showed growth of organisms. If the clinical profile does not correlate with the test results, it may be argued that the result of the test is likely to be false positive.

Correlation of test results with severity of illness in children with LRI

Of the 31 children who had evidence of lower respiratory tract infection, blood culture was positive in 4 patients. The organisms isolated were Coagulase negative *staphylococcus*, *Haemophilus influenzae*, *Candida species* and Nonfermenting gram negative bacilli (species not identified).

The DNA Macrochip showed positive isolates in 19 cases and a total of 28 organisms were identified. Of these, six samples showed multiple isolates - two organisms each in four samples and three organisms each in two samples. Concordance was present between the blood culture and DNA Macrochip in one positive sample, which was *Candida*.

Table 11. **Organisms isolated by the DNA Macrochip in children with LRI**

Organism	Number	Percentage
Klebsiella pneumoniae	4	14.3

Pseudomonas aeruginosa	4	14.3
Streptococcus pneumoniae	4	14.3
Staphylococcus aureus	4	14.3
Streptococcus species	4	14.3
Candida species	4	14.3
Aspergillus	2	7.1
Acinetibacter baumanii	2	7.1
Total	**28**	**100**

All the organisms isolated by the DNA Macrochip can cause lower respiratory tract infections, especially in the setting of febrile neutropenia.

Table 12. **Correlation of results with severity of illness in children with LRI**

	DNA Macrochip Positive (n=19)	**DNA Macrochip Negative** (n=12)	**Blood culture Positive** (n=4)	**Blood culture Negative** (n=27)
Mean duration of hospital stay (days)	9.3	5.9	12.8	11.2
Received upto 1st line antibiotics	9 (47.4%)	6 (50%)	1 (25%)	14 (51.9%)
Received upto 2nd line antibiotics	2 (10.5%)	5 (41.7%)	1 (25%)	6 (22.2%)
Received upto 3rd line antibiotics	8 (42.1%)	1 (8.3%)	2 (50%)	7 (25.9%)
Received antifungals	7 (36.8%)	3 (25%)	2 (50%)	8 (29.6%)
Outcome (Mortality)	1 (5.2%)	1 (8.3%)	2 (50%)	-

There was no statistically significant difference in outcome parameters between DNA Macrochip positive versus negative as well as blood culture positive versus negative in this group except for the use of 3rd line antibiotics between the DNA macrochip group (p= 0.04) .

We then compared DNA Macrochip positive group with blood culture positive group for the same parameters. The mean difference in hospital stay between the two groups was 3.5 days with a 95% CI between -7.2 to 14.2 and a p value of 0.5. This suggests that the mean duration of stay in the DNA Macrochip group was statistically as high as that in the blood culture positive group.

Comparing the proportion of patients who needed therapy with 3rd line antibiotics among the blood culture and DNA Macrochip positive groups the p value was 0.22. While comparing the outcome between the two groups, the p value was 0.30.
This may suggest equivalence between the 2 groups. However, more sample size will be needed to conclusively state equivalence.

Correlation of results with severity of illness in chidren with mucositis

The presence of mucositis, by itself is not a definite evidence of infection, since the chemotherapeutic agents can cause mucositis. However mucositis is well known to predispose colonization and bacteremia in the immunocompromised host. Twent one children had mucositis; the blood culture was positive in four patients. The organisms isolated were *Coagulase negative staphylococcus* (2)*, Pseudomonas aeruginosa* (1), *E Coli* and *Klebsiella pneumonia* in one patient.

The DNA Macrochip isolated 20 organisms from 15 patients, with multiple isolates in five.

Concordance was present between the blood culture and DNA Macrochip in two patients. These were *Pseudomonas aeruginosai* and *Klebsiella pneumonia*

Table 13. **Organisms isolated by the DNA Macrochip in children with mucositis**

Organism	Number	Percentage
Candida species	8	40 %
Acinetobacter baumanii	3	15 %
Klebsiella pneumoniae	3	15 %
Pseudomonas aeruginosa	2	10 %
Staphylococcus aureus	2	10 %
Streptococcus species	1	5 %
Enterococcus species	1	5 %
Total	**20**	**100**

Table 14. **Correlation of results with severity of illness in children with mucositis**

	DNA Macrochip Positive (n=15)	**DNA Macrochip Negative** (n=6)	**Blood culture Positive** (n=4)	**Blood culture Negative** (n=17)
Mean duration of hospital stay (days)	10	5.3	6.8	9.1
Received upto 1st line antibiotics	4 (26.7%)	4 (66.7%)	1 (25%)	7 (41.2%)
Received upto 2nd line antibiotics	6 (40%)	-	-	6 (35.3%)
Received upto 3rd line antibiotics	5 (33.3%)	2 (33.3%)	3 (75%)	4 (23.5%)
Received antifungals	6 (40%)	2 (33.3%)	2 (50%)	8 (53.3%)

Outcome (Mortality)	1 (6.7%)	-	1 (25%)	-

There was no statistically significant difference in outcome parameters between DNA Macrochip positive versus negative as well as blood culture positive versus negative in this group .

We then compared DNA Macrochip positive group with blood culture positive group for the same parameters. The mean difference in hospital stay between the two groups was 3.2 days and the p value was 0.51. This suggests that there is no statistically significant difference between the two groups.

Comparing the proportion of patients who needed therapy with 3rd line antibiotics among the blood culture and DNA Macrochip positive groups the p value was 0.82.

While comparing the outcome between the two groups, the p value was 0.30. This may suggest equivalence between the 2 groups. However, more sample size will be needed to conclusively state equivalence.

Correlation of results with severity of illness in children with gastro intestinal tract infections

Of the 17 children who had gastroenteritis, the blood culture was positive in one patient. The organism isolated in the blood culture was Candida. The stool culture had grown *Salmonella* and *Aeromonas* in another patient.

The DNA Macrochip isolated 18 organisms from 11 patients with six samples showing multiple isolates - two organisms each in five samples and three organisms in one sample. The organisms isolated by the DNA Macrochip are given in table 15.

The sample which grew Candida on the blood culture was reported as positive on the DNA Macrochip also, thereby showing 100% concordance with the positive culture in this group.

Table 15. **Organisms isolated by the DNA Macrochip in children with gastro intestinal tract infections**

Organism	Number	Percentage
Acinetobacter baumanii	3	16.7 %
Candida species	4	22.2
Pseudomonas aeruginosa	2	11.1
Klebsiella pneumoniae	1	5.6
Staphylococcus aureus	3	16.7
Streptococcus species	3	16.7
Bacteroides	1	5.6
Aspergillus species	1	5.6
Total	**18**	**100**

Table 16. **Correlation of results with severity of illness in children with gastro intestinal tract infection**

	DNA Macrochip Positive (n=11)	**DNA Macrochip Negative** (n=6)	**Blood culture Positive** (n=1)	**Blood culture Negative** (n=16)
Mean duration of hospital stay (days)	6.6	7.2	18	6.1
Received upto 1st line antibiotics	6 (54.5%)	4 (66.7%)	-	10 (62.5%)
Received upto 2nd line antibiotics	3 (27.3%)	1 (16.7%)	-	4 (25%)
Received upto 3rd line antibiotics	2 (18.2%)	1 (16.7%)	1 (100%)	2 (12.5%)
Received antifungals	3 (27.3%)	-	1 (100%)	2 (12.5%)
Outcome (Mortality)	-	-	-	-

There was no statistically significant difference in outcome parameters between DNA Macrochip positive versus negative as well as blood culture positive versus negative in this group .

We then compared DNA Macrochip positive group with blood culture positive group for the same parameters.

Of the patients with positivity on the DNA Macrochip, the mean duration of hospitalization was 6.6 days, as against the overall mean of 6.2 days. The t test was not applicable as there was only one patient in the blood culture positive group. Comparing the proportion of patients who needed therapy with 3rd line antibiotics among the blood culture and DNA Macrochip positive groups the p value was 0.16. This may suggest equivalence between the 2 groups, if larger numbers are studied.

All the children in both the groups survived.

Correlation of results with severity of illness in children with local infections

Of the 11 children who had local infections, three patients had cellulites and four each had ear and throat infections.

The blood culture grew in two samples;*Pseudomonas aeruginosa* and Gram negative bacilli . Pus culture grew *Pseudomonas aeruginosa* in another child.

The DNA Macrochip isolated 8 organisms from 7 patients with one sample showing multiple isolates.

Table 17. **Organisms isolated by the DNA Macrochip in children with local infections**

Organism	Number	Percentage
Acinetobacter baumanii	5	62.5 %
Streptococcus pneumoniae	1	12.5 %
Staphylococcus aureus	1	12.5 %
Streptococcus species	1	12.5 %
Total	**8**	**100**

There was no concordance between the blood culture positives samples and the DNA Macrochip in this group.

In the sample which grew Gram negative bacillus the corresponding sample analysed using the DNA Macrochip was positive *for Acinetobacter baumanii*, which is a Non fermenting gram negative bacillus.

Table 18. **Correlation of results with severity of illness in children with local infections**

	DNA Macrochip Positive (n=7)	**DNA Macrochip Negative** (n=4)	**Blood culture Positive** (n=2)	**Blood culture Negative** (n=9)
Mean duration of hospital stay (days)	11.3	12.8	3	13.7
Received upto 1st line antibiotics	3 (42.9%)	-	-	3 (33.3%)
Received upto 2nd line	2	2	2	2

antibiotics	(28.6%)	(50%)	(100%)	(22.2%)
Received upto 3rd line antibiotics	2 (28.6%)	2 (50%)	-	4 (44.4%)
Received antifungals	2 (28.6%)	-	-	4 (44.4%)
Outcome (Mortality)	-	-	-	-

There was no statistically significant difference in outcome parameters between DNA Macrochip positive versus negative as well as blood culture positive versus negative in this group.

We then compared DNA Macrochip positive group with blood culture positive group for the same parameters. The mean difference in hospital stay between the two groups was 8.3 days and the p value was 0.37. This suggests that there is no statistically significant difference between the two groups. Comparing the proportion of patients who needed therapy with 3rd line antibiotics among the blood culture and DNA Macrochip positive groups the p value was 1.0. This may suggest equivalence between the 2 groups. However, more sample size will be needed to conclusively state equivalence.

All children recovered from their infection.

Table 19. **Comparison of results with the presence of foci of infection**

Focus of infection	**DNA Macrochip positive**	**DNA Macrochip negative**	**Blood Culture positive**	**Blood culture negative**
LRI	19 / 31 (61.3%)	12 / 31 (38.7%)	4 / 31 (12.9%)	27 / 31 (87.1%)
Mucositis	15 / 21	6 / 21	4 / 21	17 / 21

	(71.4%)	(28.5%)	(19%)	(80.1%)
GIT infections	11 / 17 (64.7%)	6 / 17 (35.3%)	1 / 17 (5.9%)	16 / 17 (94.1%)
Other infections	7 / 11 (63.6%)	4 / 11 (36.4%)	2 / 11 (18.2%)	9 / 11 (81.8%)

This table shows that in more than 60% of cases with various foci of infection, DNA macrochip identified various organisms. However, blood culture positivity was less than 20% in all these cases, which is similar to published data.

DISCUSSION

In this study we aimed to evaluate the efficacy of DNA macrochip, a new PCR based technology, in early detection of septicaemia in children undergoing cancer chemotherapy in our centre. Blood culture and DNA macochip tests were done 121 children on 157 occasions.

The incidence of septicaemia in our study was 9.6%. This is less than our previous observations[44] and those published in the literature by Erten et al (20%)[55] and Dubey et al (36%)[56].

The spectrum of organisms isolated in blood culture were *Coagulase negative Staphylococcus*(4), NFGNB(3), *Pseudomonas aeruginosa*(2), *alpha hemolytic*

streptococci (1), E coli(1), *Enterococci* (1), *Hemophilus influenzae* (1), *gram negative bacilli* (1), *aerobic spore forming organism* (1), *Klebsiella pneumonia* (1) and *Candida* (1) with equal proportion of gram negative and gram positive infections. This observation was similar to that of Erten et al[55], where the proportion of Gram positive and Gram negative bacteremia was similar. However, in the study conducted bt Dubey et al[56], they found a higher proportion of Gram negative bacteremia in 83% of cases.

An obvious focus of infection was present in 79 of 157 cases; these were lower respiratory tract infection in 31, oral mucositis 21, acute gastroenteritis 17 and local infections such as cellulitis, ear infection and throat infection in 11.

This incidence is similar to that reported by Jimeno A et al[57] in a study conducted on adult patients with febrile neutropenia, where the incidence of pneumonia was 23%, acute gastroenteritis 12.8% and ENT infections 7.7%. They found a higher incidence of oral mucositis (23%), in their group.

DNA Macrochip test identified various microbes in 85 /157 samples (54.%) The spectrum of organisms isolated were *Acinetobacter baumanii* (25), *Candida* (25) *Pseudomanas aeruginosa* (14), *Streptococcus species* (other than *Streptococcus pneumonia*)(12), *Klebsiella pneumoniae* (11), *Staphylococcus aureus* (9), *Streptococcus pneumonia* (8), *Aspergillus species* (8), *Enterococcus*(5), *Bacteroides* (2), *Neisseria meningitides* (1).

This higher rate of isolation of organisms may be because of previous subclinical infections, cross reaction of the primer used in the technique for PCR amplification with the genome of other organisms, or due to contamination of the samples with

organisms. However, when we compared the corresponding reports of the blood culture and DNA Macrochip on the same sample, in only four cases both tests was positive for the same organism.

The sensitivity and specificity of the DNA Macrochip in detecting bacteremia calculated based on these observations showed 26.7 % sensitivity and 47.37 % specificity. The positive and negative predictive values were 5.4% and 84.13% respectively. These were too low for this test to be used as a diagnostic tool based on our study

Since this is a novel technique, studied for the first time in febrile infections in children on cancer chemotherapy, there were no similar studies available for comparison. However, when compared with other PCR based techniques which work on similar principle i.e. PCR amplification followed by hybridization, it is seen that the sensitivity and specificity of the DNA Macrochip are very low. Tissari et al[58] conducted a similar study on 2107 positive blood culture samples using the DNA Microarray technique and reported a sensitivity and specificity of 94.7 and 98.8% respectively.

Since blood culture, the gold standard for detection of sepsis, has a sensitivity of only around 70%, and from ours' and other's experience that only in a third of cases it is positive, we decided to compare DNA macrochip and blood culture results with clinical evidence of infection in our study population (Table 19). In 60% of children with LRI, DNA macrochip test was positive compared to 13% in blood culture positives. In case of mucositis 71% was DNA macrochip test positive as compared to 19% blood culture positivity. Those who had GIT infection 64% of cases had DNA macrochip positive

compared to 6% blood culture positive. Similar observation was made in cases of local infection; 63% DNA macrochip positive compared to 18% blood culture positive. Though more organisms were identified by DNA macrochip method, these results should be interpreted with caution at this stage, since sensitivity and specificity of this test is too low for it to be used as a diagnostic tool.

Tables 12, 14, 16 and 18 where we compared severity of illness in those with positive DNA macrochip and those with positive blood cultures, the mean duration of hospital stay, need for treatment with 3rd line antibiotics and the overall survival was statistically equivalent among the two groups. However, considering the limited sample size of our study, it is not possible to conclusively state that there is correlation between the two groups in terms of clinical profile.

Among children with oral mucositis, *Candida* was the organism most often identified by the DNA Macrochip, which is the organism which is commonly associated with mucositis. Of the eight children who were positive for *Candida* on the DNA Macrochip, four children had received empirical antifungal therapy. It may be argued that this is an indicator of higher sensitivity of the DNA Macrochip in isolating organisms, which are missed by the blood culture. However, since the number of samples is small, it is difficult to conclusively say that this observation is due to the higher sensitivity of the DNA Macrochip.

In the subgroup where we compared the results of the DNA Macrochip analysis of blood samples spiked with known organisms from pure cultures, in order to validate the

findings of the study, it was observed that the frequency of isolation of organisms were similar to that in the original study.

While there was concordance between the blood culture and DNA Macrochip reports in four out of the 15 positive blood cultures in the main study, in the standardized group there was concordance in four out of the 14 samples analysed.

Interestingly, it was observed that though there was absolute concordance in only four out of the 14 samples, in the six samples which showed isolates of multiple organisms atleast one of the organisms isolated by the DNA Macrochip showed concordance with the organism actually present in the sample. The fact that the DNA Macrochip is identifying organisms other than those actually present in the sample may be due to cross reaction between the probes used in the technique with the genome of other organisms. Also, considering the fact that the DNA Macrochip is a PCR based technique which can theoretically detect as few as 10 to 100 copies of bacterial genome in clinical samples, contamination of samples with DNA of other organisms also have to be strongly considered.

However, since the sample size in this study is small, we can only make observations regarding these possibilities and cannot conclusively comment on the reason for the false positivity or negativity of the results.

SUMMARY AND CONCLUSIONS

- We studied 157 febrile episodes in 121 febrile children undergoing chemotherapy for various malignancies and compared the results of blood culture and the DNA Macrochip in this group.
- Blood culture was positive in 15 cases whereas DNA macrochip identified 120 organisms from 85 blood samples with multiple isolates in 28 cases. Both tests identified same organism only in 4 cases.
- Sensitivity and specificity of the DNA Macrochip was calculated using blood culture as gold standard. The sensitivity was 26.7% and specificity was 47.37%, positive predictive value was 5.4% and negative predictive value was 85.13%. These were too low for a diagnostic test, therefore DNA Macrochip concept in it's present stage is not an useful methodology for the detection of septicemia.
- In the standardization study where 14 blood samples spiked with known organisms were sent to XCyton lab for DNA Macrochip analysis, it was found that the DNA Macrochip identified organisms in all 14 samples including 4 negative controls. In eight samples correct organism was detected, but in four samples additional organisms were isolated. The possibilities of the DNA primer used for PCR amplification cross reacting with the genome of other organisms, thereby giving false positive results as well as the contamination of the blood samples may explain this observation.
- When we did a comparative analysis of the blood culture and DNA Macrochip against foci of infection and severity of illness, it was found that in 60% of cases with foci of infection was positive by DNA test and the organisms identified are

known to cause these infections. Severity of illness was similar in both groups, suggesting equivalence between the two groups. However, sample sizes were too small to draw any conclusions from this.

LIMITATIONS OF THE STUDY

- This study has addressed a limited group of critical febrile neutropenic patients. Studies including larger numbers of patients and those with

different clinical conditions are necessary to validate conclusively this diagnostic methodology.

- Another limitation of the study was the reliance on traditional blood culture, which does not have 100 % sensitivity, as the gold standard for comparison.

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Annexure 1

DNA Macrochip : Test procedure

It is a Nucleic Acid Detection test which detects the virulence genes of the pathogens that cause the syndrome.

The virulent gene for each organism is identified and *Insilco* designing of primers and probes for the amplification and identification/detection of the gene has been accomplished.

These primers and probes so designed have been tested on known standard organisms that have been cultured.

Once the sensitivity and specificity was established, limit of detection of the primers were established proven clinical samples were tested.

The test involves the following process

Sample processing & Extraction of Nucleic Acid (DNA)

- 2ml of EDTA whole blood sample obtained from patients is processed to concentrate the bacteria & fungi.
- To 200 µl of the concentrated sample a 100 µl XDLB- lysis buffer is added and incubated at 37^0C for one hour. This ensures that the cell walls of all the microorganisms are lysed effectively and the nucleic acid is released.
    - After one hour incubation 20 µl of proteinase K solution is added, vortexed and incubated at 56^0C in a dry bath for 20 minutes. The proteinase K will help in scavenging all the cellular proteins that are released after the cell lysis.
    - At the end of 20 minutes, 200 µl of ethanol is added and vortexed to mix the solutions.
    - The contents of the tube are then transferred in to QIA amp spin column. The column is placed inside a clean collection tube and centrifuge at 8000 RPM for 1 minute.
    - The filtrate obtained is discarded.
    - The column is washed twice using the wash buffer. Discard the filtrate.
    - Give an empty spin to the column to remove the leftover traces of wash buffer from the column.
    - Transfer the spin column in a fresh collection tube and 200 µl of elution buffer. Incubate for 1 minute at room temperature and then centrifuge at 8000rpm for 1minute.
    - The DNA so extracted is labelled and stored at -20^0C until further use.

Amplification of Nucleic acid:

- Nucleic acid Amplification mix contains the following
    - PCR Buffer
    - Deoxy Nucleotide Triphosphate (dNTP's)
    - $MgCl_2$
    - UDG Buffer
    - Uracil DNA Glycosylase (UDG) enzyme
    - Taq polymerase

- Forward primers (mixture of 16 primers)
- Reverse Primers (mixture of 16 primers)

- To 45 µl of the amplification mix 5 µl of the extracted nucleic acid is added and allowed to amplify in a thermal cycler for 35 cycles.

Analysis of the amplified product:

Since multiplex nucleic acid amplification has been carried out on the test sample, it becomes difficult to analyse the amplified product by the conventional method of gel electrophoresis. Hence XCyton has developed a SES platform where the amplified product is detected by hybridization to the specific probes.

SES Platform has a device in which the probes complimentary to the genes amplified are pre embedded. This device is either stored at room temperature or in the refrigerator.

Hybridization protocol

- The refrigerated device is preheated in an oven set at 60^0C for 30 minutes.
- The amplified product is denatured chemically by using NaOH solution.
- The denatured product is then added to 1ml hybridization buffer and applied onto the device.
- The device is incubated at 60^0C for 1 hour.
- After one hour the hybridization buffer is discarded and the device is washed using wash buffer 3 times, incubating each time for 3 minutes at 60^0C.
- On completion of the wash device is rinsed with conjugate buffer. 1ml of 1:100 concentration of conjugate (enzyme) is added and incubated for 30 minutes.
- At the end of 30 minutes the conjugate is discarded and the device is washed with wash buffer thrice. A final rinse is given with 1XPBS.
- 1 ml of substrate solution is added to the device and incubated for 15 minutes.
- At the end of 15 minutes the solution is discarded and the device is rinsed with Milli Q water and dried.

- Once the device is dried. Coloured signal in the form of spots will be seen in the places where the amplified gene has hybridised with its complimentary probe.
- Using the template provided, based on the position of the coloured signal the organism can be identified.

TEMPLATE

K.pneumoniae	Salmonella spp	A.baumannii	H.influenzae
P.mirabilis	Leptospira	B.fragilis	N.meningitidis
P.aeruginosa	Aspergillus spp	Candida spp	S.pneumoniae
S.aereus	Streptococcus spp	Enterococcus spp	S.pyogenes
			Internal control

Quality Control Measures

The quality control measures that are followed at XCyton are as per the requirements set in CLIA/CLIS and CAP regulations. As per these guidelines do validation of the technology/assay/product is done for the following.

- Accuracy
- Precision
- Sensitivity – Limit of detection
- Analytical Specificity –Assay detects only the analyte of interest detected
- Reportable range
- Recovery of template
- Internal control

In addition to this there is also a control plan for ensuring that the testing of samples and reporting of the results are error free. Checks are done for

- Pre-analytic variables

- Analytic variables
- Results reporting
- Interpretation of testing results

Handling and transport

- Blood collected in EDTA tubes should be transported upright and secured in a screw cap container or in a rack in a transport box.
- Cushion or suspend vials during transport over rough terrain to prevent lysis of red cells. They should have enough absorbent paper around them to soak up all the liquid in case of a spill.
- In case the transport time is longer than 6-12 hours then the specimen should be sent in refrigerated condition (4-8^{0}C).

SOP for Receipt samples & Documentation of results:

The samples that are received for testing are given an identification number and entered into a log book. The final result of the sample is also documented in the same log book.

Sample identification is assured through all applicable phase of analysis including sample receipt, nucleic acid extraction, nucleic acid amplification, electrophoresis, DNA Macro chip analysis, photography & storage.

DNA Macro Chip is interpreted independently by at least two qualified persons using an objective method laid out.

The Chips are photographed and the chips are stored.

SOP of Testing

The whole analyses of the sample are done in well demarcated laboratory areas.

LABORATORY DESIGN

- **Area 1** – Specimen preparation Area (Special DNA Extraction Hoods)

Clinical samples or culture are processed and the extraction of the nucleic acid is done in this designated laboratory. Each clinical sample is extracted independently and the extracted DNA is labelled legibly and kept in an order in the racks.

- **Area 2** – Amplification (Setting up) Area in a Laminar Flow Hood
  The reagents required for amplification are prepared here and aliquoted into the required number of tubes (decided by the number of reactions that are being set). The tubes are closed and labelled legibly. Placed in a mini-cooler and taken for addition of template DNA
- **Area 3** – DNA addition area
  The extracted DNA template is added into the already prepared amplification mixture. Only one sample DNA and its corresponding amplification mix, is opened at a time.
  The vials are then put in to the thermal cycler for amplification.
- **Area 4** – DNA MACRO CHIP work up/Analysis area
  The analysis of the amplicon is done in this laboratory which is situated in a floor above the other three area .

  The flow of work is from Area 1 to Area 4. People who enter the analysis area will never enter any other work area unless he/she has had a shower (With washing of hair) and change of clothes.

GENERAL PRINCIPLES OF NUCLEIC ACID AMPLIFICATION TEST

- Each test run will have the following controls
  - **Positive Control**: will indicate the efficiency of the primers and other reagents used for amplification. In a chip since there is more than one organism that is being tested, the positive control DNA will be changed alternately.

- **Negative Control – Reagent Control**: All the reagents that are used in amplification and the water used for dilution of the reagents, is added into a tube and allowed to amplify. This will give the indication that all the reagents are contamination free and the result obtained is only due to the amplification of the DNA in the test sample.
- **Negative Control – Extraction Control**: water for PCR or a known negative sample is extracted similar to that of the samples. Amplified and analysed similar to that of the sample. If the extraction control has does not have amplified DNA then it's implied that the result is due to the amplification of the DNA in the test sample.
- **Internal Control** – a control is used from extraction through post amplification. If the internal control spot is seen, this gives an indication that the extraction & amplification have gone well.

- The test is accepted only when the following intensity score in the specific and internal control signal is seen
  - Internal Control: should have a intensity of 1 and above
  - Positive Control: should have a minimum intensity of 2
  - Negative control: No spots

  If the results do not match the specifications then the test has to be repeated

SOP For Contamination Control

DNA contamination is monitored in different areas by wipe test, using the regular detection for testing. Corrective actions are taken if contamination is detected and are documented.

Annexure 2

Proforma for study on the use of DNA Macrochip for detection of bacteremia in paediatric oncology patients with febrile illness

Study No : Date :

Name : Hospital No :

Age : Sex :

Address :

Diagnosis :

History

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Fever							
URI							
LRI							
Mucositis							
Throat							
Diarrhea							
Vomiting							
UTI							
Ear discharge							
Local infection							
CNS infection							

Number of days of fever prior to admission :

Temperature at admission :

Maximum temperature during hospital stay :

Total duration of hospital stay :

Investigations

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Hb							
TC							
DC							
Platelets							
CRP							
Blood Culture							
Other Cultures							
Others							

Previous cultures within the past 6 months

Treatment given

1st line : Ceftriaxone / Gentamicin	X	days
2nd line : Cefipime / Augmentin	X	days
3rd line : Meropenam / Vancomycin	X	days
Antifungal	X	days
G-CSF	X	days
Other antibiotics	X	days

Outcome of treatment :

Final diagnosis :

Annexure 3

PATIENT INFORMATION AND ASSENT FORM

Introduction:

We are conducting a study to detect whether a simple test using a device called DNA macrochips could help us to find out the cause for fever and severity of the illness in children with cancer who have fever and decreased blood counts. This test may help to differentiate among children who have bacterial infection, much earlier than the commonly used method called as the blood culture which takes atleast 48 hours to give a result.

Study procedure:
If you give consent to conduct this study on your child, the doctor will collect an extra blood sample along with the relevant investigations which were being done routinely here. The child will be given the same treatment irrespective of whether you give consent for the test or not.
Two ml of blood will be collected along with the routine sampling without any extra needle pricks.

Confidentiality:
The child's name will not appear on the study records.

Participation in the study:
Your participation in the study is entirely voluntary. You may refuse to take part in the study or you may stop your participation in the study at any time. This decision will not affect your treatment in this hospital.

Informed consent:
We would like to ask you whether you have understood the explanation and if you have any concerns. We would like you to participate in this study. If you agree please sign or put your thumbprint on this document below.

Participant's statement:
I have read this consent form and have discussed with the Doctor the procedures described above. I have been given the opportunity to ask questions and, which have been answered to my satisfaction. I understand that any questions that I might have asked will be answered verbally or, if I prefer, with a written statement.
I will be informed of any new findings developed during the course of this research study.

I understand that participation in this study is entirely voluntary. I understand that I may refuse my child to participate in this study. I also understand that if, for any reason, I wish my child to discontinue from the study, I will be free to do so. This will have no affect on the future care or treatment by my physician or this hospital.

I understand that medical care will be provided to my child in the event of undue side effects of this study.

If I have any questions concerning my rights as a research subject in this study, I may contact the IRB in CMC, Vellore or the Human Investigation Review Committee at 617.636.7512.

I have been fully informed of the above-described study with its risks and benefits and here by consent to the procedures set forth above.

I understand that as a participant in this study our child's identity and medical records and data related to this research study will be kept confidential, except as required by the law and except for the inspections by the study supervisor.

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Date Participant's signature

I have fully explained to the nature and purpose of this above described study and the risks that are involved in its performance. I have answered all questions to the best of my ability.

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Date Investigator's signature